SIGMA GENOSYS

SPOTs Protein Mapping Technologies

Applications

SPOTs peptide synthesis technology enables you to generate the enormous sequence diversity of custom peptide libraries for mapping applications. This technology enables you to easily identify the functional domains of proteins by conducting functional assays on peptide libraries attached to cellulose membranes1-3. The technology has applications for screening the binding specificity of any target molecules, such as proteins, peptides, nucleic acids and metals.

SPOTs technology was originally developed as a system for easily determining the amino acid sequence of a peptide antigen4-20. Peptide sequences corresponding to the specificity of an antibody are identified by conventional enzyme-linked or autoradiographic detection methods. SPOTs technology has since progressed to include a variety of new applications for research in the areas of immunology, pharmacology, enzymology, molecular biology, peptide chemistry and cell biology, which include:

- Combinatorial peptide library 22-24
- Protein-protein and receptor-ligand interactions25-321
- Phosphorylation studies33-36
- Metal binding studies37-39
- Nucleic acid binding37
- Allergen determination

Sigma Genosys offers a SPOTs kit for you to synthesise your own custom peptides as well as a custom service where Sigma Genosys will synthesise your peptides for you.

The SPOTs Kit

The SPOTs kit enables researchers of any discipline to easily synthesise their own custom library of overlapping or variant peptides on a pre-derivatised cellulose membrane.

The format of the kit offers:

- Precision - No possibility of obtaining false positives that are unrelated to your protein interest
- Simplicity - Self indicating chemistry and software package for generating a synthesis schedule
- Time saving - Coupling cycles of 90 minutes enables synthesis of 96 peptides in just three days
- Economy - Synthesis is economical with low amino acid requirements and membrane reusability
- Flexibility - Synthesis is compatible with L, D, and unnatural amino acids for analogue design

The kit is provided with an IBM/PC compatible software package that generates a variety of synthesis schedules for the following epitope analyses:

- SPOTscan - allows synthesis of overlapping peptides with specified lengths within a given protein for epitope scanning
- SPOTsizing - for determining the precise length and the boundary residues of an epitope
- SPOTsalogue - for investigating the effect of substituting amino acids within an epitope with any amino acid (L, D or unnatural)
- SPOTsalot - enables synthesis of multiple copies of specific peptides, or different peptides of various lengths

Specifications

The SPOTs kit is provided with an 8 x 12 cm cellulose membrane derivatised with a dimer of b-alanine-NH2 groups that provides the equivalent of a 6 atom linker between the membrane and the peptide. The membrane contains 96 blue spots which correspond to derivatised areas where peptide synthesis occurs on free NH2 groups.

The kit is provided with amino acids as Fmoc-OPfp esters which allow amino acids to be coupled with a simple protocol of repeated amino acid pipetting and washing. Amino acids are linked together by a condensation reaction between the C-terminal COOH and N-terminal NH2 groups of two amino acids in a COOH- ® NH2-terminus direction from the membrane. The coupling reaction is monitored visually by staining the free amines after each coupling cycle with bromophenol blue. The resulting peptides are covalently attatched to the membrane at their C-terminus and are not cleavable.

Disclaimer

Sigma Genosys guarantees that peptides synthesised according to the SPOTs method detailed in Sigma Genosys publications will

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Protein-protein and receptor-ligand interactions[25-32]
Phosphorylation studies[32-36]
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Flexibility	Synthesis is compatible with L, D, and unnatural amino acids for analogue design

Specifications

The SPOTs kit includes an 8 x 12cm cellulose membrane derivatized with a dimer of β-alanine-NH_2 groups, functioning as 6 atom linkers between the membrane and the peptides. The membrane contains 96 blue spots containing the free NH_2 groups on which the peptide synthesis occurs. The kit includes Fmoc-OPfp ester amino acids which allow a simple protocol of repeated amino acid pipetting and washing steps. Amino acids are linked together by a condensation reaction between the C-terminal COOH and N-terminal NH_2 groups of two amino acids in a COOH to NH_2-terminus direction from the membrane. The coupling reaction is monitored visually by staining the free amines after each coupling cycle with bromophenol blue. The resulting peptides are covalently attached to the membrane at their C-terminus and are not cleavable.

Custom SPOTs Service

Sigma-Genosys also offers a custom SPOTs service. We offer formats for any number of custom peptides to be synthesized on cellulose membranes using the same chemistry as in our SPOTs kit.

Peptide synthesis is performed by automated instrumentation and individual coupling reactions are followed by monitoring the fluorescence absorption of the Fmoc protecting group as well as colorimetric staining of the free amine on the spots. After the final cycle, peptides are N-terminally acetylated and then side chain deprotected. The fidelity of the peptide synthesis is monitored by synthesis of a standard control peptide that is probed with a known antibody. A duplicate control peptide is synthesized and cleaved from the membrane and analyzed by mass spectroscopy and HPLC for purity.

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SPECIALS

Contact your account representative to learn about our specials

E-MAIL

Peptide Product Manager:
Stacey Hoge
shoge@sial.com

Peptide Specialist:
Kim Jansa
kjansa@sial.com

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About the SPOTs Kit and Custom SPOTs Service

How many times can I regenerate the membrane and reprobe with antibody?

The membrane can be regenerated up to five times. However, care is required when handling the membrane, as it becomes fragile when wet and should be handled with forceps at all times. The integrity of the peptides on the membrane is not altered by the regeneration protocol.

How does Sigma Genosys QC the synthesis of SPOTs peptides in the custom SPOTs service?

Sigma Genosys synthesises three standard control peptides at the end of every custom SPOTs project, of which 2 positive and negative control peptides are probed with a standard monoclonal antibody. A third peptide is synthesised with a cleavable linker that enables cleavage of the peptide from the membrane for analysis with mass spectroscopy for mass determination and HPLC for purity

How do I know if coupling has gone to completion after each cycle?

When most Fmoc amino acids are added, the blue color of the spot often changes as the coupling reaction proceeds. The color change is highly variable and ranges from a distinct change to a very subtle or no change. This is due to the displacement of bromophenol blue from the free deprotected amino group as it couples to the next Fmoc amino acid in the synthesis cycle, enabling visual monitoring of the reaction. The nature of the color change is also dependent upon the amino acid being added and the sequence of the peptide. However, the true indication of whether coupling has occurred is when the membrane is stained with bromophenol blue at the end of a synthesis cycle, where all spots should stain blue. This indicates that a free amine has been generated on the end of the peptide, which can only occur after a successful coupling reaction.

The blue color disappears from some SPOTs after the methanol wash. Will this affect the next cycle?

No. Some amino acids have a lower affinity for the bromophenol blue, which may be displaced during the methanol wash. This does not affect the next coupling step in the synthesis.

How much peptide is synthesised per spot?

The amount of peptide that is synthesised is determined theoretically from calculating the nmoles of free amines available for coupling per spot and assuming a coupling efficiency of >98% per cycle. A typical synthesis is expected to yield between 5-10 nmol (6-12 µg for an average 10mer peptide).

What is the fidelity of the synthesised peptide?

The protocol uses an acetylation step at the end of each cycle to acetylate any unreacted free amines with acetic anhydride. This prevents them from coupling to any subsequent amino acids and virtually eliminates the synthesis of deletion sequences. The purity of the peptides synthesised varies for each peptide and is dependent upon sequence and length. Our analyses have determined that peptide purity is typically >70% for average 6-15 mers (by HPLC and mass spectroscopy).

What length of offset do I need?

Most peptide epitopes for antibody applications are between 3-6 amino acids in length. Hence, when working with a large protein, it is recommended that the offset does not exceed 3 amino acids in order to localise the epitope. An offset of 1 is recommended for finer mapping of the epitope.

Can HRP or AP conjugated secondary antibodies be used for epitope analysis?

We do not recommend the use of HRP or AP enzymes for colorimetric detection. The reaction products of these enzymes cannot be removed effectively from the membrane when regenerated according to the regeneration protocol. However, HRP and AP enzymes can be used with chemiluminescent detection protocols for analysis of the SPOTs membrane.

Can unnatural or modified amino acids be used in the synthesis?

The SPOTs synthesis technology is compatible with D and L forms of amino acids as well as unnatural amino acids. However, the

chemistry requires that the amino acids have an Fmoc on their N-terminus, an OPfp or ODhbt ester on their C-terminus and acid labile protecting groups on their side chains (e.g. Pmc, OtBu, Trt, tBoc, tBu).

How many peptides can I synthesise with the amino acids provided with the kit?

The quantities of amino acids provided with the kit are sufficient for approximately 600 couplings of each amino acid, which is equivalent to approximately 700 peptides, depending on amino acid usage and peptide length. The kit is provided with a membrane that enables synthesis of up to 96 peptides. Sigma Genosys also offers smaller membranes that enable up to 24, 48 and 96 peptides to be synthesised.

Can epitope analyses be conducted with polyclonal antisera?

Yes. Although polyclonal antisera contain many antibodies that recognise a variety of epitopes on a protein or peptide, only a proportion of these sequences will be antigenic. SPOTs technology can been used to identify the predominant immunogenic sequences in a peptide or protein with polyclonal antisera.

Does Sigma Genosys have a recommended control peptide that I can use to check my synthesis?

Sigma Genosys sells a mouse monoclonal antibody to the extracellular domain of human c-erb-1/EGF-R (Cat no. OM-11-951) that recognises the epitope: CAHYID.

Does any secondary structure formation occur with the peptides that are synthesised on the membrane?

Yes. Secondary structures in peptides can form in sequences as short as 6 amino acids. However, the secondary structure formed by a peptide on a SPOTs membrane may not necessarily resemble the secondary structure formed by the same peptide within the full length native protein. Hence, epitope mapping results should be interpreted with the caveat that the sequence recognised by the antibody may form a conformational epitope.

What is the stability and the appropriate storage conditions for the membrane?

The derivatised membrane is very stable prior to peptide synthesis and can be stored indefinitely when kept desiccated at -20° C. Following peptide synthesis, the membrane should be stored in identical conditions for 6-8 months, however the shelf life will significantly diminish. The stability of the peptides varies according to sequence and would be susceptible to oxidation on methionines and intramolecular bridging through cysteines.

I conducted an antibody probing experiment on my SPOTs peptides and did not observe any signal

An antibody probing experiment can fail due to a low concentration of the primary or secondary antibody, or due to loss of activity of the secondary antibody. Alternatively, the concentration of the primary antibody should be increased to about 10-15µg/ml. Some researchers use a dilution of the primary that has been optimised for western blotting applications. In some instances this is not a good predictor of the dilution that can be used for SPOTs analysis and some optimisation should be performed.

However, it is important to determine if the conjugated secondary antibody can convert substrate using the following procedure:

QC TO TEST ACTIVITY OF b GAL-CONJUGATED SECONDARY ATNIBODIES

Materials

PVDF membrane (1 x 10 cm per antibody)

20ml SPOTs substrate development solution (Section 7.7, 7.8, 7.9)

100ml PBS

100ml 80% methanol

10 microcentrifuge tubes per antibody

For each antibody

1. Take 10 microcentrifuge tubes and number each from 1-10. Add 10µl of PBS to tubes 2-10.
2. Add 20µl of the conjugated antibody solution to tube 1. Take 10µl of antibody from tube 1 and perform serial 1:2 dilutions with tubes 2-10.
3. Wet a 1 x 10cm strip of PVDF paper with 80% methanol (20ml, 3 mins) followed by equilibration in PBS (50ml, 5 mins). Remove PVDF paper from PBS, drain excess PBS and place on a small sheet of Whatman 3MM paper (10 x 20 cm).
4. Apply 5µl from each tube (1-10) on equidistant spots along the strip of PVDF and allow to dry.

5. Wash the membrane in 50ml PBS for 5 mins, remove excess PBS and apply the substrate development solution.

Secondly it is important to determine if the secondary antibody is recognising the primary antibody using the following procedure:

QC TO TEST b GAL-CONJUGATED SECONDARY ANTIBODIES FOR RECOGNITION OF PRIMARY ANTIBODY

Materials

PVDF membrane (1 x 10 cm per antibody)

20ml SPOTs substrate development solution (Section 7.7, 7.8, 7.9)

100ml PBS

100ml 80% methanol

10 microcentrifuge tubes per antibody

Blocking buffer

Secondary antibody

For each antibody

1. Take 10 microcentrifuge tubes and number each from 1-10. Add 10µl of PBS to tubes 2-10.
2. Add 20µl of the primary antibody solution to tube 1. Take 10µl of antibody from tube 1 and perform serial 1:2 dilutions with tubes 2-10.
3. Wet a 1 x 10cm strip of PVDV paper with 80% methanol (20ml, 3 mins) followed by equilibration in PBS (50ml, 5 mins). Remove PVDF paper from PBS, drain excess PBS and place on a small sheet of Whatman 3MM paper (10 x 20 cm).
4. Apply 5µl from each tube (1-10) on equidistant spots along the strip of PVDF and allow to dry.
5. Wash the membrane 2 times in 50ml PBS for 5 mins, remove excess PBS, apply 20ml 1X solution of blocking foam buffer and incubate for 30min.
6. Wash the membrane 2 times in 50 50ml PBS and apply a 1:200 dilution (10ml, PBS) of the secondary antibody and incubate for 45mins
7. Remove the PBS solution and apply the substrate solution to the membrane.

After probing the SPOTs membrane, numerous random SPOTs were positive.

SPOTs peptides can appear positive if the primary or secondary antibody are used at a greater that optimal concentration. High concentrations of antibodies can result in non-specific interactions with peptides. Hence, reducing the concentration of the primary antibody and using a standard concentration of the secondary (1:200) normally alleviates this problem.

I am preparing my reagents to begin SPOTs peptide synthesis and I am uncertain about whether I need to purify my DMF and NMP.

The reagents detailed in section 2.2 of the SPOTs instruction manual are needed prior to commencing synthesis. It is very important to make certain that the DMF and NMP are of *"biosynthesis"* or *"peptide synthesis"* grade with minimal free amine content. The DMF and NMP from the suppliers we recommend do not require column purification when purchased. We recommend that 4A sieves be prepared according to the protocol detailed in section 3.1 of the SPOTs instruction manual, added to the DMF and NMP (20%v/v) in the bottle and allowed to stand for 5 days. Prior to use, the DMF and NMP should be checked for free amine content according the protocol detailed in section 4.2.

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5. Use ELISA-based methods to demonstrate binding of the ligand to the receptor to ascertain that the interaction is titratable and that it binding can be detected when one molecule is attached to a solid phase, rather than in solution. The dynamics of protein-protein interaction on a solid phase a different to those in solution.

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result in the synthesis of linear peptides with the desired sequence and attached to a cellulose membrane through C-terminal ends. Sigma Genosys does not make claims as to the ability of peptides synthesised on SPOTs membranes to perform in solid phase assays conducted by the researcher.

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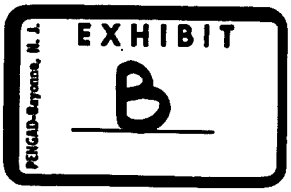
Louise N. Johnson

Structural studies with protein kinases, their inhibitors and their regulation.

Introduction

Our major interests are in the biochemical and structural basis of molecular interactions that underlie cytoplasmic signal transduction. Almost all cellular processes are controlled by phosphorylation catalysed by protein kinases. Studies on the 27 or so protein kinase structures that have been solved to date show that they all have a common core structure but each has a distinct substrate specificity and a distinct mechanism for control. The importance of phospho-signalling pathways in eukaryotic signal transduction is demonstrated by the observation that the human genome contains 575 protein kinases, the third most populous domain in the entire proteome. We employ X-ray crystallographic methods and more recently electron microscopy (see also Section 11.2 by Dr Catherine Vénien-Bryan). Much effort is required on expression and purification methods to provide suitable quantities of proteins. We address the following themes: How is specificity achieved in molecular recognition? How do regulatory subunits exert their control? What is the structural basis for the catalytic mechanism? How can knowledge of structure be exploited in understanding drug interactions?



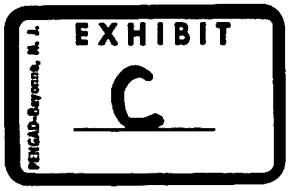
• Plant Receptor-Like Protein Kinases (RLPK)

The activity of many enzymes and receptor proteins is regulated by phosphorylation and dephosphorylation, and protein kinases (PK) are important parts of signal transduction chains. Of particular interest in plants are the **plant receptor-like kinases** (RLPK) that contain a large extracellular domain connected by a membrane-spanning stretch to the intracellular kinase domain (Fig. 1). Although little is known about their precise functions, it is very likely that they play major roles in the perception and transmission of external signals. We investigated a **RLPK** cloned from *Catharanthus roseus* and the properties of the protein expressed in heterologous hosts. This kinase autophosphorylates predominantly on threonine and little on serine. The mechanism is a strict *cis*-autophosphorylation, i.e. the protein phosphorylates only itself, not other kinase molecules (*trans*-autophosphorylation). **We succeeded in identifying by site-directed mutagenesis a specific threonine in the kinase domain that is autophosphorylated and is also necessary for the phosphorylation of substrate proteins** (Schulze-Muth et al., 1996, see Abstract below!). We are presently investigating a kinase which phosphorylates the RLPK from *Catharanthus roseus*.

Publication

- Schulze-Muth, P., Irmler, S., Schröder, G., Schröder, J.: Novel type of receptor-like protein kinase from a higher plant (*Catharanthus roseus*): cDNA, gene, intramolecular autophosphorylation, and identification of a threonine important for auto- and substrate phosphorylation. J. Biol. Chem. 271: 26684-26689 (1996).
  Accession: Z73295

  We characterize CrRLK1, a novel type of receptor-like kinase (RLK), from the plant *Catharanthus roseus* (Madagascar periwinkle). The protein (90.2 kDa) deduced from the complete genomic and cDNA sequences is a RLK by predicting a N-terminal signal peptide, a large extracytoplasmic domain, a membrane-spanning hydrophobic region followed by a transfer-stop signal, and a C-terminal cytoplasmic protein kinase with all 11 conserved subdomains. It is a novel RLK type because the predicted extracytoplasmic region shares no similarity with other RLKs. The autophosphorylation was investigated with affinity-purified proteins expressed in *Escherichia coli*. The activity was higher with Mn^{2+} than with Mg^{2+} and achieved half-maximal rates at 2-2.5 µM ATP. The phosphorylation was predominantly on Thr, less on Ser, and not on Tyr. In contrast to other plant RLK, the kinase used an intra- rather than an intermolecular phosphorylation mechanism. After protein cleavage with formic acid, most of the radioactivity was in a 14.1-kDa peptide



located at the end of the kinase domain. Mutagenesis of the four Thr residues in this peptide identified Thr-720 in the subdomain XI as important for autophosphorylation and for phosphorylation of ß-casein. This Thr is conserved in other related kinases, suggesting a subfamily sharing common autophosphorylation mechanisms.
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W0030

Mechanism of Phosphoryl Transfer in Kinases. Louis Delbaere(1), Allan Matte (2),Leslie Tari (3) & Athena Sudom (1), (1) Department of Biochemistry, University of Saskatchwan, 105 Wiggins Road, Saskatoon, SK S7N 5E5 Canada (2) Biotechnology, Research Institute, 6100 Royalmount Avenue, Montreal QC H4P 2R2 Canada (3) Department of Biological Sciences, University of Calgary, Calgary AB T2N 1N4 Canada

Kinases usually catalyse the transfer of a phosphoryl group from a nucleoside triphosphate to another substrate. These enzymes typically possess a binding site for the purine/pyrimidine base and another binding site for the triphosphoryl group(often a P-loop). Mg2+ is usually coordinated by oxygen atoms of the beta- and gamma-phosphoryl groups and a lysine side chain often bridges two oxygen atoms of the beta- and gamma-phosphoryl groups; these interactions serve to activate the NTP for phosphoryl transfer. Some kinases such as phosphoenolpyruvate carboxykinase require a second divalent metal cation for optimal activity. Phosphoryl transfer likely occurs via a direct displacement mechanism. The transition state for phosphoryl transfer may be either associative (SN2-like) or dissociative (SN1-like). The associative transition state involves a pentagonal bipyridimal structure whereas the dissociative transition state involves a planar trigonal structure. Recent crystal structures of complexes of kinases with substrates and AlF_3 provide evidence that phosphoryl transfer may proceed through both types of transition states in kinases.

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